

Survival and growth of newly transformed *Lampsilis cardium* and *Lampsilis siliquoidea* in a flow-through, continuous feeding test system

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Abstract: A test system was evaluated for assessing chronic toxicity of waterborne chemicals with early life stage mussels. To determine if the test system could result in $\geq 80\%$ survival in a control (unexposed) group, fat mucket mussels (*Lampsilis siliquoidea* Barnes, 1823) and plain pocketbook mussels (*L. cardium* Rafinesque, 1820) 1 day post transformation were stocked into test chambers (250 mL beakers, water volume, 200 mL, 21 °C, 40 mussels of 1 species per chamber) within a test system constructed for conducting chronic, continuous exposure, flow-through toxicity tests. The test system contained 60 chambers containing silica sand, 30 chambers with *L. siliquoidea*, and 30 with *L. cardium*. Each chamber in the continuous feeding system received 1 of 6 food types prepared with concentrated algal products. After 28 days, mussels were harvested from chambers to assess survival and growth. For *L. siliquoidea*, mean survival ranged from 34 to 80% and mean shell length ranged from 464 to 643 μm . For *L. cardium*, mean survival ranged from 12 to 66% and mean shell length ranged from 437 to 612 μm . The maximum mean growth rate for *L. siliquoidea* was 12.7 $\mu\text{m}/\text{d}$ and for *L. cardium* was 11.8 $\mu\text{m}/\text{d}$. When offered a continuous diet of *Nannochloropsis*, *Tetraselmis*, and *Chlorella* for 28 days in the test system, the survival of 1 day post transformation *L. siliquoidea* was 80%. The test system can be easily enhanced with a pumping system continuously delivering test chemical to the test system's flow stream allowing for chronic toxicity tests with 1 day post transformation mussels.

Key words: juvenile mussels, laboratory cultures, diet, chronic exposure

Freshwater mussels are some of the most imperiled organisms in North America. Of the nearly 300 taxa of freshwater mussel populations in North America, 70 species (23%) are listed as endangered or threatened and another 40 species (14%) are candidates for listing as endangered or threatened (Williams *et al.* 1993, Neves 1997, 2004, Graf and Cummings 2007). The causes for the declines in unionid abundance and diversity have not been well characterized. Potential anthropogenic stressors that may be contributing to the declines include siltation, dams, mining wastes, introduction of exotic bivalve species such as zebra mussels (*Dreissena polymorpha* Pallas, 1771) and Asian clams (*Corbicula fluminea* Müller, 1774), industrial wastes, agricultural point and nonpoint pollution, and pharmaceuticals and personal care products.

Recent publications have reported the sensitivity of freshwater mussels to contaminants including pesticides, ammonia, chlorine, and heavy metals (Valenti *et al.* 2005, 2006, Bringolf *et al.* 2007a, 2007b, 2007c, Wang *et al.* 2007a, 2007b). Much of the work was conducted in accordance with the American Society for Testing and Materials (ASTM 2006) Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels. The ASTM guide recommends that acute toxicity tests (<14 days) be initiated with juvenile mussels within 5 days post transformation and that chronic studies (>14 days) be conducted with mussels 60 to 120 days

post transformation. The age designation is based on high mortality of newly transformed mussels cultured for more than 7 days in laboratory settings (Gatenby *et al.* 1996, 1997, Jones *et al.* 2004). Mortality after 7 or 14 days has been attributed to an inappropriate food supply and not understanding of nutritional requirements during this life stage (Ingersoll *et al.* 2007). The conventional wisdom is that newly transformed juveniles depend on pedal feeding to obtain food, presumably using cilia on the foot to move food. Later in development, it is thought that juveniles begin to use a combination of pedal and suspension feeding, with more emphasis on suspension feeding with increased age.

The ASTM guide indicates that for acute trials (≤ 4 days) initiated with juvenile mussels <5 days post transformation, survival in the control groups is typically $>90\%$. For chronic trials (10-28 days) with mussels 2 to 4 months post transformation, survival in the control groups is typically $>80\%$. These recommendations are based on mortality during the apparent sensitive stage from 14 to 42 days post transformation. Although mussels live in sediment, the ASTM guide recommends that mussel toxicity trials be conducted with water only, presumably eliminating sediment as a potentially complicating factor in the trial.

It is generally understood that the earliest life stages of aquatic organisms are the most sensitive. As for mussels, Valenti *et al.* (2006) found that mussel sensitivity to chlorine

decreased as mussel age increased. Initiating chronic studies with 2 to 4 months post transformation mussels potentially overlooks critical life stages beginning immediately after transformation through the next 2 months. This study evaluated a test system for suitability to assess the chronic toxicity of waterborne chemicals to mussels <2 months of age. The test system was evaluated by determining survival and growth of 1 day post transformation *Lampsilis cardium* (Rafinesque, 1820) and *Lampsilis siliquoidea* (Barnes, 1823), after 28 days of culture with 1 of 6 food sources. This test system will be beneficial by generating chronic toxicity data necessary to ensure that water quality criteria for individual chemicals are set or revised to protect all aquatic organisms, including early life stage mussels.

MATERIAL AND METHODS

Experimental animals

Largemouth bass (*Micropterus salmoides*; $N = 48$; length, 8–13 cm) were equally distributed to 12 flow-through 38-L aquaria. Five days later, *Lampsilis cardium* glochidia were removed from adult mussels (acquired from Pool 7 of the Mississippi River, Wisconsin) by prying open the shells and flushing glochidia out with a stream of well water from a 10-mL syringe. Glochidial viability was verified by the rapid closure of shells when glochidia in a subsample were exposed to a sodium chloride solution (ASTN 2006). All glochidia were mixed in 1 common container. Twenty four largemouth bass were infested with glochidia by transferring groups of 4 fish to about 2 L of aerated well water containing an aliquot of the glochidial slurry. About 5 min later, fish were returned to their respective aquaria. The same procedures were repeated with adult *Lampsilis siliquoidea* mussels.

Exposure system

The system was constructed with materials and operated with parameters described in the standard guide for conducting toxicity trials with freshwater mussels (ASTM 2006). The test system was set up in an enclosed chamber where incandescent lighting provided 16 hours of light and 8 hours of darkness with 20 minute transition periods for lights to fade on and off. Test chambers ($N = 60$) were supported on platforms elevated over a fiberglass tank. Test chambers were 250-mL glass beakers with a 12-mm hole drilled in the bottom of each beaker. A hole was drilled through silicone stoppers and glass standpipes inserted into each stopper which were inserted into the beaker holes. The height of standpipes was set for a water volume of 200 mL. Nitex® bolting cloth (161 μm mesh; mention of trade or manufacturer name is solely for providing specific information and does not imply endorsement by the U.S. Geological

Survey) covered standpipe openings inside the beakers. Stoppers, standpipes, and bolting cloth were secured with silicone sealant. Silica sand (blend FW 120, Badger Mining Corporation, Berlin, Wisconsin) was sieved to a common size range (75 to 150 μm) using a Retsch® sieve shaker (F. Kurt Retsch GmbH & Co., Germany) and USA standard testing sieves (Soiltest Inc., Evanston, Illinois). Silica sand (25 ± 1 g) was dispensed into each test chamber.

Distribution boxes (1 box for each food type) delivered food solutions to the test chambers through polyethylene tubing (PE160), and were mounted above the test chambers. A head box was mounted above the distribution boxes. Flow of well water from the head box to distribution boxes (186–201 mL/min) was controlled by a 3-way polycarbonate stopcock (1 for each distribution box). Food solutions entered their respective flow streams above the distribution boxes through a second polycarbonate stopcock. The nominal flow rate from distribution boxes to the test chambers was 10 mL/min.

Food preparation and food delivery

All food types were prepared with Reed Mariculture (Campbell, California) concentrated products and did not contain live organisms. Each food type was prepared daily in 2-L volumetric flasks. After adding the prescribed volumes of concentrated products, flasks were filled with about 250 mL of well water, swirled, and allowed to stand for about 1 min to prevent cytolysis before the process was repeated at least 4 more times. Thereafter, the flask volumes were adjusted to 2 L with well water.

Food type 1 was prepared with 2.3 mL of *Nannochloropsis* 3600 Instant Algae® (a marine green microalga). Food type 2 was prepared with 1.2 mL of *Nannochloropsis* 3600 Instant Algae and 1.2 mL of *Tetraselmis* (a marine large green flagellate). Food type 3 was prepared with 1.2 mL of *Nannochloropsis* 3600 Instant Algae, 0.6 mL of *Tetraselmis*, and 0.8 mL of marine *Chlorella* (a marine green microalga). Food type 4 was prepared with 1.2 mL of *Nannochloropsis* 3600 Instant Algae and 2.8 mL of *Thalassiosira weissflogii* (a large marine diatom). Food type 5 was prepared with 1.2 mL of *Nannochloropsis* 3600 Instant Algae and 2.4 mL of *Pavlova* (a small golden brown flagellate). Food type 6 was prepared with 1.2 mL of *Nannochloropsis* 3600 Instant Algae, 1.6 mL of *T. weissflogii*, and 1.2 mL of *Pavlova*. At about 2 weeks, a layer of organic material accumulated on top of the sand in most test chambers. Ingredients were reduced by one half throughout the remaining 2 weeks of the study.

Food solutions were continuously mixed on stir plates while being delivered (1 mL/min) to the test system through Masterflex® Tygon® L/S 13 LFL (through the pump head) and Masterflex C-Flex® L/S 13 (spliced to the LFL tubing after the pump head and connected to the polycarbonate

stopcock above the distribution box) tubing using a Masterflex® Digi Staltic pumping system (Cole-Parmer, Vernon Hills, Illinois). Flows of food solutions through the test system were initiated 1 day before mussels were stocked into test chambers.

Collection and distribution of juveniles

Each day during the infestation period, the bottoms of aquaria were siphoned to remove detritus and newly transformed mussels. Sixteen days after infesting fish with *Lampsilis siliquoidea* glochidia, newly transformed *L. siliquoidea* were collected by filtering siphoned water through a 153- μ m sieve made with Nitex bolting cloth. The following day, groups of 20 mussels displaying foot movement were randomly stocked into each of 30 chambers until each chamber had 40 mussels. Concurrent with stocking the test chambers, 1 day post transformation mussels ($N = 16$) with foot movement were stored in a glass vial filled with 10% buffered formalin for 1 day, then transferred to 70% ethanol.

Newly transformed *Lampsilis cardium* were collected 17 days after infesting fish with *L. cardium* glochidia. The following day, 30 test chambers were stocked according to the procedures previously described and an additional 22 1-day post transformation mussels were stored in 10% buffered formalin, then 1 day later, transferred to 70% ethanol.

General husbandry

Each day, water temperature, dissolved oxygen, and pH were measured and water flow to each chamber was verified or measured. Three times during the study, light intensity was measured directly over the chambers. Once per week, water alkalinity and hardness were measured in water samples taken from the head box.

Mussel collection and shell length measurement

Twenty eight days after stocking each species, the contents of each chamber were sieved through a 202 μ m sieve. The material collected on the sieve was rinsed into a petri dish. Live and dead mussels were enumerated with the aid of a dissecting microscope. Mussels were considered alive if foot movement or ciliary activity was observed. Live mussels from each chamber were enumerated, transferred to 10% buffered formalin for 1 day then transferred to 70% ethanol.

Shell length (the maximum distance across the shell parallel to the hinge) was measured from a digital photomicrograph of the mussel (QImaging Micropublisher 3.3 RTV digital camera, QImaging, Canada; Nikon Eclipse model E600 microscope, Nikon Corporation, Melville, New York). Shell length was measured using image analysis software (Image Pro® Express, ver. 6.0.0.319, Media Cybernetics, Silver Spring, Maryland) that was calibrated from a photomicrograph of a stage micrometer.

Data analyses

Each food type was delivered to 5 test chambers with *Lampsilis cardium* and 5 chambers with *L. siliquoidea*. Each chamber was randomly assigned to 1 of 10 blocks so that each food type was represented in each block (a randomized block design in a 2×3 configuration). Feed type 1 was labeled as the control diet for statistical comparisons. Each side of the test system (30 chambers on each side) was assigned to 1 of the 2 species.

Percent survival was determined by comparing the number of live mussels found with the number of mussels stocked into each chamber. Growth rate was determined by subtracting the mean shell length of the 1 day post transformation mussels from the shell length of mussels collected at the end of the trial. The difference was divided by the trial duration (28 days) to calculate a daily growth rate.

The mussels could experience one of two outcomes, survival or death. Survival and death are therefore binary random variables that presumably follow Bernoulli distributions. The response data may be categorized as either the numbers of survivors or the numbers of dead, out of a fixed number of mussels in each test chamber. The numbers of survivors or dead, therefore, follow a binomial distribution. In this analysis, the focus was on the proportional risk of survival versus death. Test chambers were the fundamental experimental units. Mussels held in the same chamber experienced identical environmental conditions but those conditions may have varied randomly among chambers within the same treatment group. Mussels were continuously offered various food solutions for 28 days. Therefore, food solution was considered a categorical (classification) variable rather than a continuous variable in the data analysis because each food solution contained a different algal species or combination. The probability of death at day 28 was modeled by a generalized linear mixed model fitted using SAS GLIMMIX (Wolfinger and O'Connell 1993). The correlation between the two types of outcomes for the test chamber population was modeled with shared (G-side) random effects with food solution assignment as a grouping variable. Over dispersion within the model was modeled using an R-side covariance structure. Determination of significant differences between feed types was accomplished by comparison of least-square means.

Shell length (μ m) and growth rate (μ m/day) were determined only for mussels that survived until the end of the study. Data were evaluated to ensure assumptions of normality (Shapiro and Wilk 1965). Shell length and growth rate data as a function of food solution were fit to a generalized linear model (McCullagh and Nelder 1989) using SAS GLM, and model parameters and 95% confidence intervals were estimated using maximum-likelihood estimation. Likelihood-ratio F -tests were used to test hypotheses about the relation

between shell length or growth rate and food solution. Determination of significant differences between feed types was accomplished by pair-wise comparison of least-square means using a Bonferroni adjustment for multiple comparisons. All statistical analyses were performed with SAS software (SAS 2003) and analyses considered to be significant if $P \leq 0.05$.

RESULTS

Physical parameters of the test system

The mean water temperature throughout the trial was 20.8 °C. Mean dissolved oxygen concentrations for each food type for each species ranged from 7.77 to 7.96 mg/L. Mean pH for each food type for each species ranged from 7.32 to 7.34. Mean alkalinity was 107 mg/L as CaCO_3 and mean hardness was 167 mg/L. Light intensity measurements over the test chambers ranged from 40 to 160 lux. Mean flow rate through test chambers was 9.9 mL/min with individual measurements ranging from 87.0 to 10.8 mL/min.

Recovery of mussels from test chambers and mussel survival

The mean recovery of live and dead *Lampsilis siliquoidea* from each test chambers was >87% (Table 1). Survival of *L. siliquoidea* ranged from 33.5 to 80.0% among all food types (Table 1). Food type explained a significant amount of the variation in *L. siliquoidea* survival (Chi-square test, $\chi^2 = 8.40$, $df = 5$, $P < 0.01$). Food type 3 resulted in the greatest percent survival although survival resulting from food types 1 and 4 were not statistically different. Survival variability with those

food types were 13, 20, and 8.7% relative standard deviation (RSD), respectively. Comparing food type 3 and 4 (each with similar survival and variability $\leq 20\%$ RSD), mussels fed food type 3 were 1.3 times more likely to survive than if fed food type 4.

The mean recovery of live and dead *Lampsilis cardium* from each test chamber was >83% (Table 1). Survival for *Lampsilis cardium* ranged from 12.0 to 66.0% among all food types (Table 1). Food type explained a significant amount of the variation in *L. cardium* survival ($\chi^2 = 6.06$, $df = 5$, $P < 0.01$). Food type 1 resulted in the greatest percent survival although survival generated by food types 2, 3, and 4 were not statistically different. Only food type 3 with a survival of 60.5% had a variability value of <20% RSD.

Mussel growth

The mean shell length of 1 day post transformation *Lampsilis siliquoidea* was 287 μm (5.4% RSD). *Lampsilis siliquoidea* mean shell lengths after 28 days ranged from 464 to 643 μm among all the food types (Table 2). Food type explained a significant amount of the variation in *L. siliquoidea* growth rate ($\chi^2 = 4,517$, $df = 5$, $P < 0.01$). Shell lengths of *L. siliquoidea* offered food type 3 were significantly greater than shell lengths resulting from any other food type. Growth rates for *L. siliquoidea* ranged from 6.3 to 12.7 $\mu\text{m}/\text{d}$ and were significantly greater in mussels offered food type 3 than in mussels offered any other food type (Table 2).

The mean shell length of 1-day post transformation *Lampsilis cardium* was 283 μm (5.9% RSD). Mean shell lengths of *L. cardium* after 28 days ranged from 437 to 612 μm among all the food types (Table 2). Food type explained a

Table 1. Recovery and survival of *Lampsilis siliquoidea* and *Lampsilis cardium* after stocking the test system with mussels 1 day post transformation and offering mussels 6 feed types continuously for 28 days. Each species was represented with 30 chambers, 40 mussels per chamber, each chamber receiving 1 of 6 feed types. Mean values of the same species denoted with a common letter are not significantly different. SD, standard deviation; RSD, relative standard deviation.

Species	Food type	Mean recovery (%)	SD (%)	RSD (%)	Mean survival (%)	SD (%)	RSD (%)
<i>Lampsilis siliquoidea</i>	1	83.0	14	17	67.1 ^{a,b}	13	20
	2	83.5	16	20	56.5 ^{a,c}	19	34
	3	88.0	11	13	80.0 ^b	11	13
	4	91.0	8.9	9.8	74.8 ^b	6.5	8.7
	5	88.5	4.2	4.7	42.5 ^{a,c}	27	63
	6	89.5	12	13	33.5 ^c	12	35
<i>Lampsilis cardium</i>	1	84.5	14	16	66.0 ^a	16	23
	2	84.0	13	16	43.4 ^{a,c}	22	50
	3	79.0	8.0	10	60.5 ^a	6.5	11
	4	84.0	7.0	8.3	51.0 ^a	26	52
	5	81.0	15	18	12.0 ^b	12	99
	6	89.0	5.2	5.8	23.5 ^{b,c}	9.8	42

Table 2. Shell lengths and growth rates of *Lampsilis siliquoidea* (mean shell length at 1 day post transformation, 287 μm) and *Lampsilis cardium* (mean shell length at 1 day post transformation, 283 μm) after stocking the test system with mussels 1 day post transformation and offering mussels 6 feed types continuously for 28 days. Each species was represented with 30 chambers, 40 mussels per chamber, each chamber receiving 1 of 6 feed types. Means of the same species denoted with a common letter are not significantly different. *SD*, standard deviation; *RSD*, relative standard deviation.

Species	Food type	N	Mean shell length (μm)	<i>SD</i> (μm)	<i>RSD</i> (%)	Min (μm)	Max (μm)	Mean growth rate ($\mu\text{m}/\text{d}$)
<i>Lampsilis siliquoidea</i>	1	124	495 ^{a,d}	79	16	334	697	7.4
	2	104	464 ^b	66	14	323	642	6.3
	3	152	643 ^c	102	16	335	870	12.7
	4	144	509 ^a	70	14	360	681	7.9
	5	35	471 ^{b,d}	63	13	360	595	6.6
	6	55	485 ^{a,b}	70	14	344	623	7.1
<i>Lampsilis cardium</i>	1	109	512 ^a	75	15	278	676	8.2
	2	73	437 ^b	49	11	350	596	5.5
	3	102	612 ^c	86	14	395	785	11.8
	4	91	535 ^d	57	11	386	628	9.0
	5	20	510 ^{a,d,e}	70	14	372	628	8.1
	6	43	476 ^e	62	13	329	616	6.9

significant amount of the variation in *L. cardium* growth rate ($\chi^2 = 4,240$, $df = 5$, $P < 0.01$). Shell lengths of *L. cardium* offered food type 3 were significantly greater than shell lengths resulting from any other food type. Growth rates for *L. cardium* ranged from 5.5 to 11.8 $\mu\text{m}/\text{d}$ and were significantly greater in those mussels offered food type 3 than in mussels offered any other food type (Table 2).

DISCUSSION

After 28 days in the test system, *Lampsilis siliquoidea* proved to be more rugged (max. survival, 80%) than *Lampsilis cardium* (max. survival, 66%). Statistically, *L. siliquoidea* specimens were about 2.3 times more likely to survive than *L. cardium*. Both species responded similarly to any particular food type: *i.e.*, if one species responded poorly to a particular food type, the other species did as well.

Growth rates attained in this study (*Lampsilis siliquoidea*, 12.7 $\mu\text{m}/\text{d}$; *Lampsilis cardium*, 11.8 $\mu\text{m}/\text{d}$) were consistent with growth rates previously reported. Barnhart (2006) reported lampsiline growth rates ranging from 4.7 to 12.2 $\mu\text{m}/\text{d}$ in a recirculating laboratory system with cultured algae as a food source. Gatenby *et al.* (1997) measured growth rates of 3.9 to 10.4 $\mu\text{m}/\text{d}$ through a 140-day period for *Villosa iris* (I. Lea, 1830) held in aerated dishes and fed various diet combinations of cultured algae and silt. Juvenile *Lampsilis fasciola* (Rafinesque, 1820) had growth rates of about 15 $\mu\text{m}/\text{d}$ through a 105 or 112-day period when held in a recirculating system and fed cultured algae (Steg 1998). Newton *et al.* (2003) also reported *L. cardium* growth rates ranging from

4.2 to 7.2 $\mu\text{m}/\text{d}$ for laboratory reared mussels. Newton *et al.* (2003) measured growth rates after mussels (3 to 5 days post transformation) were held in well water at 22 °C with no food source for periods of 4 and 10 days.

In our test system, feed type significantly affected shell length and growth rate for each species. As with survival, shell length and growth rate response of each species was similar for a given food.

The recovery of live and dead mussels from individual test chambers of each species ranged from 60 to 100%. Several factors may have contributed to mussel recoveries less than 100%. First, mussels may have been compromised during the stocking process when handled with a pipette at least twice and agitated several times with swirling and rinsing. If mussels were damaged to the point of causing early mortality, then the shells from those dead mussels may not have been discernable at the end of the study for enumeration. The shells from newly transformed mussels are relatively fragile and may have either deteriorated through time or disintegrated during reconnaissance procedures at the end of the study.

There were a few cases where mussel recovery was >100%. A plausible reason for the errant recovery may have been the counting of 1 shell from a recently dead mussel as 1 whole mussel. The orientation of the shell in the petri dish may not have allowed for discerning whether the shell was a whole mussel or only 1 of 2 shells separated during the recovery procedures. When more than 40 mussels were recovered, adjustments were made in the recovery and survival calculations. Although considered to be a conservative approach, this method of calculating recovery and survival did not substantially affect the data.

Most systems for rearing newly transformed mussels in laboratory settings have been designed for propagation rather than for conducting chronic toxicity tests (Gatenby *et al.* 1996, 1997, Jones and Neves 2002, Jones *et al.* 2004, Barhnhart 2006, Kovitvadhi *et al.* 2006, 2008). Our test system was instead designed for rearing newly transformed mussels while conducting chronic (≥ 28 day) toxicity trials, and can be easily enhanced with a pumping system to continuously deliver chemical solutions allowing chronic toxicity assessments of waterborne chemicals. In the test system, survival of 1 day post transformation *Lampsilis siliquoidea* after 28 days was 80%, the typical minimum survival for 2-4 month old mussels in a control treatment group during toxicity tests conducted for 10 to 28 days (ASTM 2006).

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